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Two-color multiplex assay for the identification of orthopox viruses with real-time LUX- PCR

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Abstract

The LUX™ [Light Upon eXtension] is a real-time detection system that can be used for the detection and quantification of pathogens nucleic acids. In this study we used a universal LUX™ approach, a variation of the LUX™ detection system, for identifying *Orthopoxvirus* nucleic acids in real time. This approach enables the design of sequence-specific primer sets in high identity genome sequences. The assay described here is designed to allow simultaneous detection of *Variola* and other orthopox viruses in a multiplex format, with a limit of detection in the range of 50–100 copies of the *Orthopoxvirus* genome. Regression analysis showed that the assay was linear over seven orders of magnitude, with 0.97 correlation coefficient. The sensitivity and specificity of the assay, as determined from a panel of 100 samples that contained nucleic acids from a variety of bacteria and viral species, were rated at 98%. Thus, the assay offers a sensitive and specific tool for simultaneous identification and quantification of *Variola* and other orthopox viruses, and the approach allows more flexible sequence-specific primers design for pox viruses as well as other microbial pathogens.

Keywords: Orthopoxvirus; LUX-PCR; Real-time PCR; Multiplex assay

1. Introduction

Members of genus *Orthopoxvirus* (Family *Poxviridae*) contain a double-stranded DNA genome that replicates in the cell cytoplasm. The most important member of this genus is the *Variola* virus, the agent of the now eradicated smallpox. Other orthopox viruses, such as monkeypox, cowpox, buffalopox and *Vaccinia* can also infect humans causing smallpox-like disease [1–10].

Several Real-time PCR methods for detecting orthopox viruses with high levels of sensitivity and specificity have been developed [11–18]. These reports used either SYBR green intercalating dye which has limited specificity or probes with a fluorophore and a quencher moieties, which could increase the cost of the assays.

The LUX™ (Light Upon eXtension) is PCR-based detection system that uses one self-quenched fluorogenic primer labeled with a single fluorophore. Typically 20–30 bases long, the oligonucleotide primer is designed with the fluorophore close to the 3′end in a hairpin structure. The fluorophore may be one of several commonly used dyes such as FAM [(5 or 6-carboxyfluorescein), TET (6-carboxy-4′, 5′-dichloro-2′,4,7,7′-tetrachlorofluorescein), JOE (6-carboxy-4,5-dichloro-2,7-dimethoxyfluorescein) or others. This configuration and the presence of a guanine or cytosine at the 3′ end of the oligonucleotide intrinsically renders fluorescence quenching capability so that a separate quenching moiety is not needed. Upon incorporation and extension of the fluorescein-labeled primer during the PCR, the primer emits fluorescence whose intensity is proportional to the amount of amplified nucleic acids, and can be measured in real time by using a real time fluorescence detection instrument [19].

In this study, we used a universal LUX™ approach, a variation of the LUX™ detection system, for identifying *Orthopoxvirus* nucleic acids and distinguishing between the *Variola* virus and other orthopox viruses in real time in a single reaction tube. This approach enables the design of specific primers in sequences with high identity without following the design rules that apply to standard

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14. ABSTRACT
The LUXtrade mark [Light Upon eXtension] is a real-time detection system that can be used for the detection and quantification of pathogens nucleic acids. In this study we used a universal LUXtrade mark approach, a variation of the LUXtrade mark detection system, for identifying Orthopoxvirus nucleic acids in real time. This approach enables the design of sequence-specific primer sets in high identity genome sequences. The assay described here is designed to allow simultaneous detection of Variola and other orthopox viruses in a multiplex format, with a limit of detection in the range of 50-100 copies of the Orthopoxvirus genome. Regression analysis showed that the assay was linear over seven orders of magnitude, with 0.97 correlation coefficient. The sensitivity and specificity of the assay, as determined from a panel of 100 samples that contained nucleic acids from a variety of bacteria and viral species, were rated at 98%. Thus, the assay offers a sensitive and specific tool for simultaneous identification and quantification of Variola and other orthopox viruses, and the approach allows more flexible sequence-specific primers design for pox viruses as well as other microbial pathogens.

15. SUBJECT TERMS
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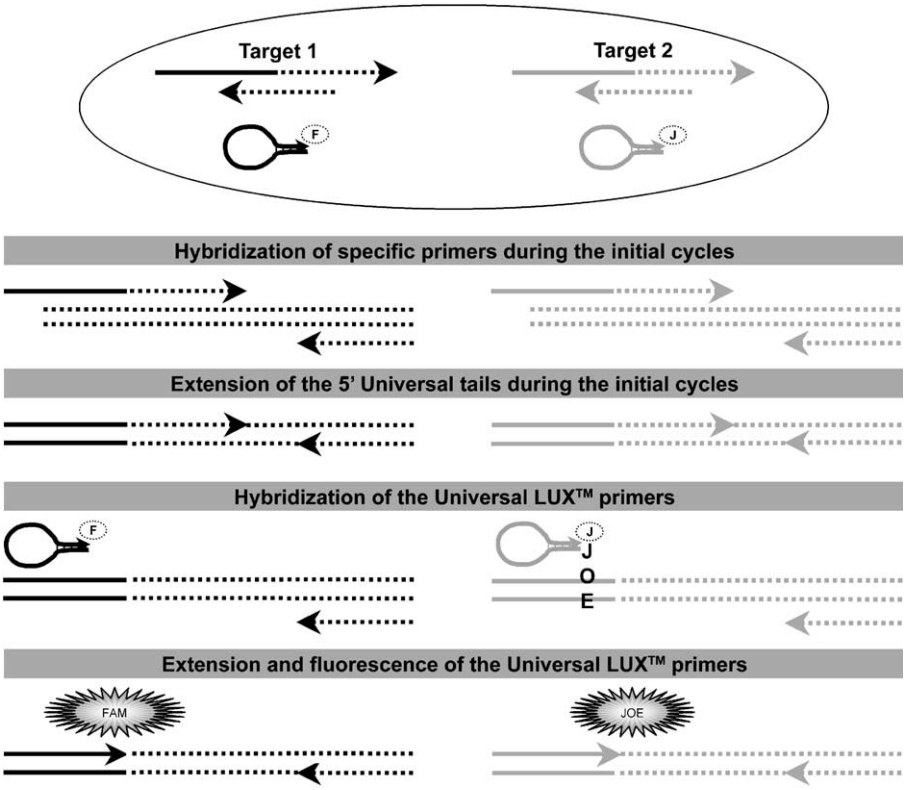

Fig. 1. Universal LUX™ in multiplex mode. A set of specific primers are designed for each of two targets. In each pair, one of the primers has a 5′ end extension that has the same sequence as one of the 2 Universal LUX™ primers. In the initial cycles, only the specific primers are extended. After a few cycles, the Universal LUX™ primers are hybridized and produced Light Upon eXtension.

LUX™ primer design. This system consists of a labeled LUX™ primer with a unique sequence, designed not to hybridize with any sequences within known genomes. The Universal primer is coupled to gene-specific primers to amplify target sequences. The forward gene-specific primer is synthesized with a 5′ extension identical to the sequence of the Universal LUX™ primer. During PCR, the extended primer anneals to the target sequence and is extended by DNA polymerase. The reverse unlabeled primer is then extended and the complementary sequence is synthesized, including the sequence complementary to the Universal LUX™ primer. In the later cycles, the Universal LUX™ primer anneals to this newly created sequence and is extended, generating a fluorescent signal (Fig. 1). Using two different Universal sequences, one labeled with a FAM dye and the second labeled with a JOE dye, this system can be used to design multiplex detection assays.

2. Materials and methods

2.1. Universal LUX™ primers

Two Universal LUX™ primers were designed with different fluorophores, FAM or JOE. Both primers were designed to be roughly the same length, and have the same G+C content and melting temperature. Primers were blasted against public databases to confirm that they do not recognize sequences within unrelated genomes. The sequences of these Universal LUX™ primers are proprietary of Invitrogen, and are commercially available (Invitrogen, Carlsbad, CA).

2.2. Unlabeled primers

To amplify all orthopox virus DNA, we used ALL-FORWARD: 5′-TCATCTGGAGAATCCACAACA-3′and ALL-REVERSE: 5′-CAGTGTCTGTGACTGTATGAT-CTTC-3. To specifically amplify *Variola* DNA, we used VAR-FORWARD: 5′-AACAGACAAGACGTCGGG-3′ and VAR-REVRSE: 5′-GTCTGTGACTGTATGATCTT-CTTTATT-3′. These specific primer sets were designed after analyzing all published hemagglutinin genes of orthopox viruses and using the criteria for standard real-time PCR primers design, including the primers length, Tm, GC contents, self-complementarity, amplicon size and minimizing primer-dimer formation. On each forward primer a 5′ extension sequence corresponding to the proprietary sequences of the Universal LUX™ primer was added. All primers were synthesized by Invitrogen.

2.3. DNA samples

All viral and bacterial DNA samples used in this study were from the collections of the United States Army Medical Research Institute of Infectious Diseases and the Centers for Disease Control and Prevention, and are summarized in Table 1. DNAs included isolates of camelpox, cowpox, monkeypox, myxoma, tanapox, rabbitpox, raccoonpox, skunkpox, and *Vaccinia* viruses. The origins, propagation, and harvesting procedures for orthopox viruses are described in Ropp et al. (1995) [20]. DNA was extracted from virus-infected cells, virions, and scabs by using the Aquapure DNA kit (Bio-Rad). Prior experiments demonstrated that the material was noninfectious after 60 min of incubation at 55 °C in Aquapure lysis buffer.

2.4. RNA samples

Total RNA was extracted from virus-infected cell cultures by the TRIzol LS reagent (Invitrogen) according to the manufacturer's instructions. RNA pellets were dissolved in 5–10 μL of Rnase-free water with 40U of RnaseOUT recombinant inhibitor (Invitrogen) and stored at −80 °C until used.

2.5. Statistical analysis

Regression analysis was done by using Microsoft Excel to evaluate linearity and determine the quantitative performance of each assay.

2.6. Real-time quantitative PCR

The reactions were carried out in 30 μL volumes on the SmartCycler [Cepheid Inc., Sunnyvale, CA]. Each reaction consisted of 15 μL of Platinum® quantitative PCR SuperMix—uracil DNA glycosylase (UDG). Final concentration of constituents was 20 mM Tris [pH 8.4]; 50 mM KCL; 0.2 μM of each dATP, dCTP, dGTP; 0.4 μM dUTP; 5 mM of $MgCl_2$; 0.45 U of Platinum Taq DNA polymerase; 0.3 U of UDG; stabilizers; and an additional 0.9 U of Platinum Taq DNA polymerase; 40 nM of each extended forward primers; 200 nM of each reverse primer; 200 nM of each Universal LUX™ primer; additives [final concentration 0.2 mM Tris–HCL [pH 8.0], 0.2 mg/ml of bovine serum albumin, 150 mM of trehalose, 0.2% Tween 20], and 2 μL of template DNA.

PCR amplification was performed in a single tube on the SmartCycler as follows: one cycle of 50 °C for 2 min, one cycle of 95 °C for 2 min, followed by 45 cycles of 95 °C for 10 s and 60 °C for 45 s. The melting curve analysis was performed by denaturation at 95 °C for 10 s, followed by a step at 50 °C for 30 s. and heating to 80 °C with a rate of 0.1 °C/s, while monitoring the fluorescence in the temperature ramp. Fluorescence detection of *Orthopoxvirus* DNA

Table 1
Cross-reactivity results of the *Variola*/*Orthopoxvirus* multiplex assay

Species/sample	Strain/isolate	Conc. [fg]	*Variola* LUX	*Orthopoxvirus* LUX
Poxvirus samples				
Camelpox	Somalia	1000	0/2	2/2
Cowpox	Brighton	1000	0/2	2/2
Monkeypx	Zaire 1996-I-16	1000	0/2	2/2
Monkeypx	Zaire 1996-I-16	100	0/5	5/5
Monkeypx	Zaire 1996-I-16	10	0/6	5/6[a]
Myxoma	CDC	1000	0/2	0/2
Rabbitpox	CDC	1000	0/2	2/2
Racconpox	CDC	1000	0/2	2/2
Skunkpox	CDC	1000	0/2	0/2
Tanapox	CDC	1000	0/2	0/2
Vaccinia	BHS	1000	0/2	2/2
Vaccinia	CPN	1000	0/2	2/2
Variola J7R	BSH	10	6/6	6/6
Variola J7R	BSH	1	7/7	7/7
Variola J7R	BSH	0.1	12/12	12/12
Bacterial samples				
Bacillus anthracis	4728	1000	0/2	0/2
Clostridium perfringens	13124	1000	0/2	0/2
Francisella tularensis	NA	1000	0/2	0/2
Haemophlius influenzae	10211	1000	0/2	0/2
Listeia monocytogenes	15313	1000	0/2	0/2
Neisseria lactamica	23970	1000	0/2	0/2
Staphylococcus aureus	25923	1000	0/2	0/2
Stpahylococcus hominis	27844	1000	0/2	0/2
Streptococcus pyogenes	19615	1000	0/2	0/2
Yersinia pseudotuberculosis	690	1000	0/2	0/2
Viral samples				
Black Creek Canal	39179	100	0/2	0/2
Dengue 3	CH3489	100	0/2	0/2
Eastern Equine Encephalitis	FL-4679	100	0/2	0/2
Ebola	Boniface	100	0/2	0/2
Ebola	Zaire95	100	0/2	0/2
Hantaan	76-118	100	0/2	0/2
Lassa	Josiah	100	0/2	0/2
Rift Valley fever	ZH 548	100	0/2	0/2
Venezuelan Equine Encephalitis	PE-4.0904	100	0/2	0/2
West Nile	Crow 397-99	100	0/2	0/2
Yellow fever	Assibe	100	0/2	1/2[b]
Other samples				
Human genomic DNA	NA	1000	0/2	0/2
Total			100	100

NA not applicable. One hundred samples were randomly tested; 25/25 *Variola* DNA samples were correctly detected; 24/25 genomic DNA from orthopox viruses were detected; 49/50 negative samples were not detected. Therefore, the sensitivity and specificity were rated at 98%.

[a] False negative.

[b] False positive.

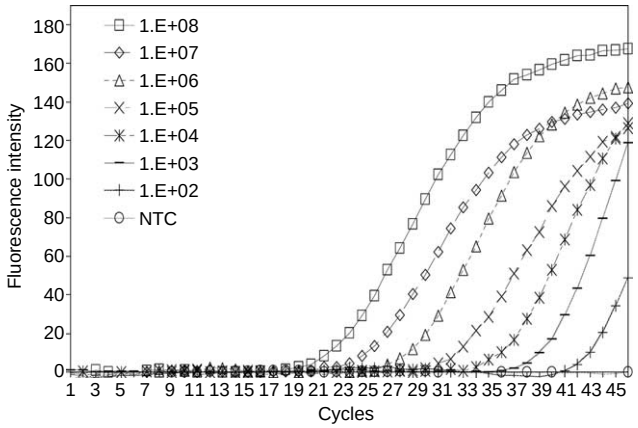

Fig. 2. Limit of detection of the multiplex assay expressed in copy numbers using monkeypox virus genomic DNA. Tenfold serial dilutions of monkeypox virus genomic DNA representing about 100–100,000,000 copies were tested. Each curve represents the mean fluorescence value of three replicates.

was monitored on the SmartCycler by using the FAM channel and detection of *Variola* DNA by using the TET channel.

3. Results

Assay performance characteristics were evaluated by determining the lower concentration of DNA that could be detected, the linearity of a range of different DNA concentrations and the ability of the assay to specifically identify *Variola* virus and other orthopox viruses among a panel of unrelated bacterial and viral nucleic acids samples.

To determine the assay limit of detection (LOD), serial 10-fold dilutions from *Variola* J7R cloned DNA [13] and monkeypox virus DNA representing 50–100,000,000 copies of DNA were analyzed by the two-color assay in two separate runs. The LOD of *Variola* virus J7R cloned DNA

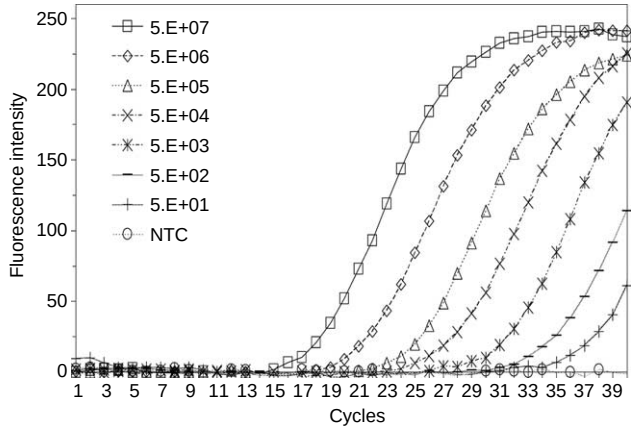

Fig. 3. Limit of detection of the multiplex assay expressed in copy numbers using cloned *Variola* J7R DNA. Tenfold serial dilutions of *Variola* J7R DNA representing about 50 to 50,000,000 copies were tested. Each curve represents the mean fluorescence value of three replicates.

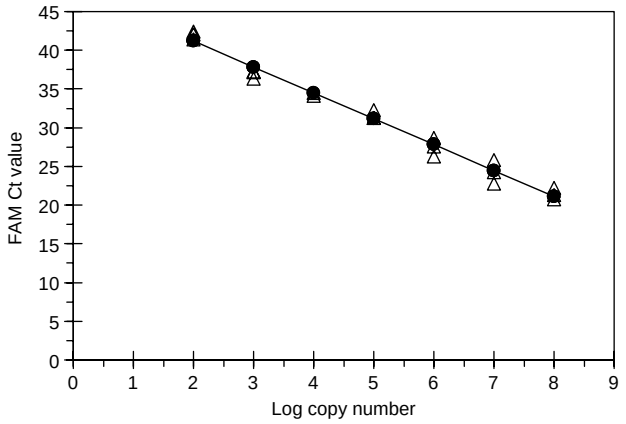

Fig. 4. Regression analysis on the C_t value against log copy numbers showing the dynamic range of the multiplex assays with monkeypox virus DNA preparations ranging from 100 to100,000,000 copies [$R^2=0.98$; significance $F=2.8\times10^{-17}$].

was approximately 50 copies (Fig. 2) and that of monkeypox virus genomic DNA was 100 copies (Fig. 3). Regression analyses showed a strong linear correlation ($R^2=0.97$) between the C_t values and DNA concentrations over seven orders of magnitude for both FAM and JOE reporters of the multiplex assay (Fig. 4, Fig. 5).

The LOD of each multiplex assay was tested for reproducibility. Of 60 test repetitions with 50 copies of the *Variola* virus J7R plasmid DNA, 59 were detected with a mean C_t value of 35.07 ± 1.86, and 58 of 60 test repetitions with 100 copies of monkeypox virus genomic DNA were detected with a mean C_t value of 34.9 ± 1.76 (Fig. 6).

The results of the sensitivity and specificity tests of the two-color assay are shown in Table 1. The *Variola* virus probe detected 25 of 25 samples containing *Variola* J7R DNA. The *Orthopoxvirus* probe detected 24 of the 25 DNA samples containing camelpox, cowpox, racconpox, *Vaccinia*, and monkeypox viruses. One false negative was obtained from samples of monkeypox DNA. Of the 50 negative samples, one

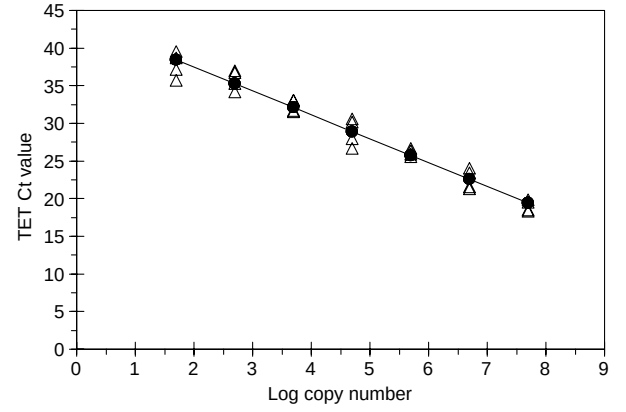

Fig. 5. Regression analysis on the C_t value against log copy numbers showing the dynamic range of the multiplex assay with cloned *Variola* J7R DNA preparations ranging from 50 to 50,000,000 copies [$R^2=0.97$; significance $F=8\times10^{-21}$].

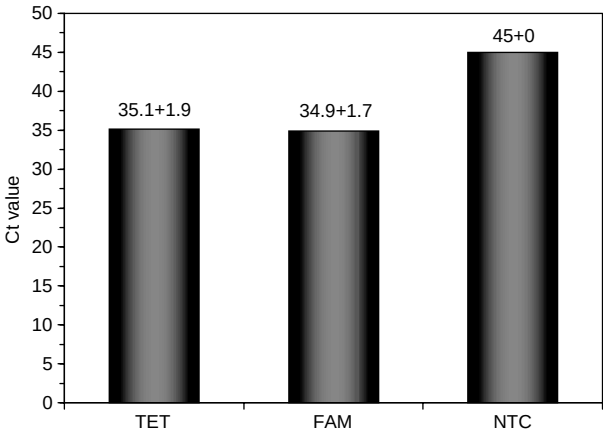

Fig. 6. Reproducibility of multiplex assays was expressed as the average C_t values of 60 repetitions. The mean C_t value and standard deviation are indicated on top of each bar.

was a false positive. Therefore, both sensitivity and specificity of this two-color multiplex assay were rated at 98%. It is worth mentioning that the false positive was obtained form a yellow fever virus samples that was unrelated to orthopox virus, suggesting that the false positive results were probably due to mishandling of the DNA samples. When the false positive and false negative samples were re-tested, both samples were correctly identified.

4. Discussion

The genomes of *Orthopoxvirus* species are extremely conserved and require a technology that can offer flexibility in primers design to enable high level of specificity for species differentiation. We developed a multiplex Universal LUX™ PCR assay for simultaneous detection and differentiation of *Variola* virus from other orthopox viruses.

Several publications have reported the use of SYBR green or dual-labeled probes for detecting orthopox viruses [11–16]. As of today, there have been no reports of single-labeled probe usage, such as in the LUX™ system, for orthopox virus diagnosis. LUX™-PCR has several advantages over SYBR green or dual-labeled probes real-time PCR. It is a cost-effective assay, more amenable to multiplexing, and allows robust melting curve analysis of PCR product. This is possible because PCR products retain the fluorescent label after extension of the LUX™ primers. The melting curve can be used to resolve problems of primer dimers and false results. Primer dimers usually have a lower melting point (75 °C) than those of PCR products (78-90 °C). Reported real-time PCR methods that collect data in the exponential growth phase such as the fluorogenic 5′ exonuclease and minor groove binding (MGB) probe-based PCR do not perform melting curve analysis. Besides the LUX™ system, the only cheaper alternative for melting curve analysis is the double-stranded DNA binding dye chemistry, such as SYBR-green. However, with this chemistry, unspecific fluorescence intercalation occurs due to non-specific amplification and primer-dimer formation. Another disadvantage of the SYBR green-based real-time PCR chemistry is the requirement for extensive optimization [21]. Other published assays that used melting curve analysis included two probes, anchor and sensor probes, and dual-labelled probes [17,18].

Our multiplex assay had a good sensitivity level and linearity with a broad dynamic range. The assay detected at least 50 copies of the *Variola* virus cloned DNA (0.1 fg) and 100 copies of monkeypox virus genomic DNA (10 fg). With this assay, it was also possible to detect less than 10 fg of DNA with 75% reproducibility rate (data not shown). However, we elected to present an LOD (10 fg) because it was highly reproducible at a rate of at least 96% (58/60 repetitions). Previous studies using non-multiplexed Real-time PCR reported LOD between 4 and 250 copies [13,15–18]. It is noteworthy that the variations in LOD among different assays depend on a number of factors, including the target sequence, the assay platform, and more importantly the purity of the DNA template. In our study, the concentration of genomic DNA used to determine LODs was determined by a spectrophotometer with an absorbance 260/280 ratio of 1.75. Although, our assay is a multiplex assay, its LOD remains within the range of LODs of non-multiplexed assays reported previously and is well below the range of viral load expected in clinical samples. For example, up to 10^6 pock-forming units or 200 pg of *Variola* DNA were obtained from skin lesions of the smallpox rash by day 2 of the rash [18,22].

The performance of the LUX™ assay was evaluated in terms of clinical sensitivity and specificity by testing 100 DNA samples that were obtained from a variety of bacterial and viral species. The assay was highly sensitive (98%) and specific (98%), indicating its utility for clinical samples testing, particularly because of its ability to distinguish between *Variola* and other *Orthopoxvirus* infections in a single tube reaction. Only one false negative and one false positive result were obtained. These two samples were unambiguously clarified first, by melting curve analysis and second by re-testing the samples three times to confirm the results.

In conclusion, the assay presented here should be helpful for rapidly identifying and assaying *Variola* and other orthopox viruses, allowing the opportunity to confirm or rule out a *Variola* infection should such an infection occur accidentally or deliberately. Furthermore, the assay can be used as a quantitative tool to assess viral loads in vaccine and anti-viral drug efficacy studies.

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